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Use of *Lactiplantibacillus plantarum* for dairy and non-dairy fermented products

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Abstract: In this study, two strains of *Lactiplantibacillus plantarum* 299v and CCDM 181 were tested for their ability to grow in milk and soy beverage, for stability during cold storage of fermented beverages, compatibility with yoghurt culture and activity against yeasts. Both strains grew better in soy drink compared to milk. During co-culturing with the yoghurt culture, sufficient acidification of milk and soy beverage necessary for the production of fermented products was achieved. The stability of tested strains in media at pH 4.5 for 28 days at 5 °C was good. *L. plantarum* was effective in the inhibition of undesirable yeast growth, but the ability was strain-specific. Tested strains demonstrated also a strain-specific ability to suppress the growth of yoghurt culture bacteria. For a possible application of co-culturing *L. plantarum* with the yoghurt culture, verification of the mutual compatibility of specific strains is necessary.

Keywords: antifungal activity; LAB compatibility; lactobacilli; soy beverage; yoghurt culture

The use of non-traditional species of lactic acid bacteria (LAB) in traditional fermented products is intensively researched due to the application of different probiotic strains or due to their protective function. Much attention has recently been paid to the species *Lactiplantibacillus plantarum*, which occurs in a variety of habitats, such as plants, food raw material (meat, fish, vegetables, milk) or fermented dairy products as well as in the gastrointestinal tracts of human and animals (Todorov and Franco 2010). *L. plantarum* produces rods, and it is a mesophilic bacterium that grows in a range of 15 °C to 45 °C. It requires nutrient-rich media but compared to other lactobacilli, it is an extremely versatile microorganism. It is adapted to very different conditions and is known for its highly variable strains possessing diverse phenotypes and larger variable genomes (Martino et al. 2016). *L. plantarum* is facultatively heterofermentative with a comprehensive carbohydrate utilisation system. It produces both important inducible enzymes (aldolase, phosphoketolase), and thus metabolises hexoses into both isomers of lactic acid, ferments pentoses to form other metabo-

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lites (acetic acid/ethanol), or can ferment them under aerobic conditions only to acetic acid and carbon dioxide. In addition to the utilisation of monosaccharides, the fermentation of sugar alcohols, oligosaccharides, or glycosides is also important (Essid et al. 2009).

The production of antimicrobial metabolites by *L. plantarum* is, similarly to other LAB, a strain-specific property. Antibacterial activity has been demonstrated against members of the family *Enterobacteriaceae*, *Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes, Pseudomonas aeruginosa* and others (Arena et al. 2016; Tremonte et al. 2017). The activity of *L. plantarum* strains against fungi and yeasts is also significant. It was confirmed in a number of studies that dealt with the suppression of the most frequently occurring fungi in food, such as *Aspergillus* spp., *Fusarium culmorum*, *Penicillium* spp. (Russo et al. 2017). The activity against yeasts is important in view of the technology of fermented products, where yeasts are the most common contaminants. Different strains of *L. plantarum* can inhibit the growth of *Debaryomyces hansenii, Kluyveromyces marxianus, Saccharomyces cerevisiae, Rhodotorula mucilaginosa* and others (Dinev et al. 2018).

Some strains of *L. plantarum* have also been proven to have a number of positive probiotic properties *in vitro* and in clinical studies (Wen-Ching et al. 2019; Toshimitsu et al. 2020), the best-documented strain being *L. plantarum* 299v (Hoppe et al. 2015). It has been shown that the species *L. plantarum* is more abundant in the gastrointestinal tract with increased consumption of vegetables, which is often absent in people with a Western diet (Yin et al. 2017).

The aim of this study is to test the possibility of applying the species *L. plantarum* to dairy and fermented soy products to verify its antimicrobial activity and compatibility with yoghurt culture strains.

MATERIAL AND METHODS

Microorganisms used. *L. plantarum* CCDM 181, a strain with proven antifungal activity (Horáčková et al. 2018) (Culture Collection of Dairy Microorganisms, Laktoflora®, Milcom, Prague, Czech Republic) and commercial probiotic strain *L. plantarum* 299v (DSM 9843) were used in this study. Other lactobacilli were obtained from culture collections: *L. plantarum* CCDM 375, *Lactobacillus delbrueckii* subsp. *bulgaricus* CCDM 31, CCDM 171, CCDM 25 and *L. plantarum* ATCC 14917. Lactobacilli were routinely cultivated in De Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at pH 5.6 for 18 h at 37 °C in a 5% v/v CO_2 atmosphere. Yoghurt culture YC-381 (Christian Hansen, Hørsholm, Denmark) was recovered from a lyophilised form in skimmed ultra-high-temperature (UHT) milk, cultivation at 30 °C, 18 h, aerobically. For agar diffusion method both *L. delbrueckii* subsp. *bulgaricus* YC-381 and *Streptococcus thermophilus* YC-381 were separated and isolated on an agar plate (MRS or M17) (Merck, Darmstadt, Germany). The streptococci used also came from a collection of microorganisms: *S. thermophilus* CCDM 31, CCDM 148 were cultivated in M17 broth or in MRS broth at 30 °C, aerobically. Yeasts *K. marxianus* CCDM 259 and *Kluyveromyces lactis* var. *lactis* CCDM 21 were cultivated in MRS broth (30 °C, pH 5.6, aerobically).

Carbohydrate utilisation and enzymatic activity. These activities were determined by the commercial API 50 CH test and API ZYM test (both BioMérieux, Marcy-l'Etoile, France). The tests were performed according to the manufacturer's instructions.

Active acidity. Active acidity was measured with a Jenway 3020 pH meter (Jenway, Staffordshire, United Kingdom) using a temperature-compensated glass electrode.

Inhibition activity. Activity against yeasts was tested by an agar diffusion method on MRS soft agar, pH 5.6, where the inoculation of yeasts of 10^4–10^5 colony forming units (CFU) mL^{-1} was used. After agar solidification, 5 µL of fresh lactobacilli culture (10^9 CFU mL^{-1}) was spotted on the surface and plates were cultivated at 30 °C for 48 h. The results are expressed as the mean of inhibition zones measured in two directions from three different determinations. The same method was used to prove the inhibition activity of *L. plantarum* against *S. thermophilus* on MRS or M17 soft agars or against *L. delbrueckii* subsp. *bulgaricus* on MRS soft agar.

Cultivation in different media. Cultivation in skimmed UHT milk (Pragolaktos, Prague, Czech Republic) or unsweetened soy beverage Bio (Delhaize, Brussels, Belgium) was performed at 30 °C, aerobically with 2% v/v inoculum. For count determination, after appropriate dilution, *L. plantarum* cells were cultivated on MRS agar (Merck, Darmstadt, Germany) (pH 5.6, 37 °C, 48 h, 5% v/v CO_2) and counted as CFU mL^{-1}. Standard ISO 7889 was followed to determine the cell number of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. The results are means of two parallel measurements in two independent cultivations (n = 4). If the number of microorganisms was determined during co-culturing, the procedure, according to Veselá et al. (2019), was used.

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RESULTS AND DISCUSSION

In the first part of the study, the activity of probiotic strains *L. plantarum* 299v and *L. plantarum* CCDM 181 against yeasts and their properties important for the technology of fermented products were first tested, i.e. the ability to grow at low pH, the ability to ferment different carbohydrates, to ferment milk and soy drink, acidification ability and compatibility with yoghurt culture microorganisms.

Characteristics of *L. plantarum* strains. Based on the results of the API 50 CH test, it was proved that both strains were capable of utilising all basic hexoses (glucose, galactose, fructose, mannose); they fermented *L*-arabinose and *D*-ribose, but they did not use ribitol; also lactose and soy oligosaccharide *D*-raffinose and sugar alcohols (mannitol, sorbitol), which are often used as sweeteners in the food industry, were utilised. The bacteria also used oligosaccharides that are not digestible in the human body (melibiose, melezitose) and glycosides (amygdalin, arbutin, salicin), but they did not ferment inulin, starch or glycogen. The utilisation of some carbohydrates may be strain-specific; these results are consistent with other studies (Essid et al. 2009).

The results of the enzyme activity showed a similarity between the two tested strains. Although some strains of *L. plantarum* can be weakly lipolytic (Papamaloni et al. 2003), the studied strains showed no lipolytic activity and only very low [less than 5 nanomoles (nmol) of substrate released] esterase activity. Moderate activity (up to 20 nmol) was detected in both strains for aminopeptidases, the strains differed in chymotrypsin-like enzyme activity, which was null in strain 299v but confirmed in CCDM 181. Both strains showed high β-glucosidase (more than 40 nmol) and β-galactosidase (30–40 nmol) activities. β-glucosidase is important for the cleavage of major soy isoflavonoids into aglycones that have biological effects (Angelotti et al. 2020).

As already mentioned, yeast is the most common contaminant of yoghurts. Defects in fermented products are often caused by species of the genus *Kluyveromyces*. Spoilage is associated with the production of mucilage, organic acids, gas or alcohol, and it has an effect on sensory properties – a change in taste or surface pigmentation (Spanamberg et al. 2014). As can be seen in Figure 1, both strains suppressed the growth of the yeast *K. lactis* var. *lactis* CCDM 271. However, when another species, *K. marxianus* CCDM 259, was used, the inhibitory activity was zero. The same yeast species were used in the study by Delavenne et al. (2013), who reported that *K. marxianus* is less sensitive to the

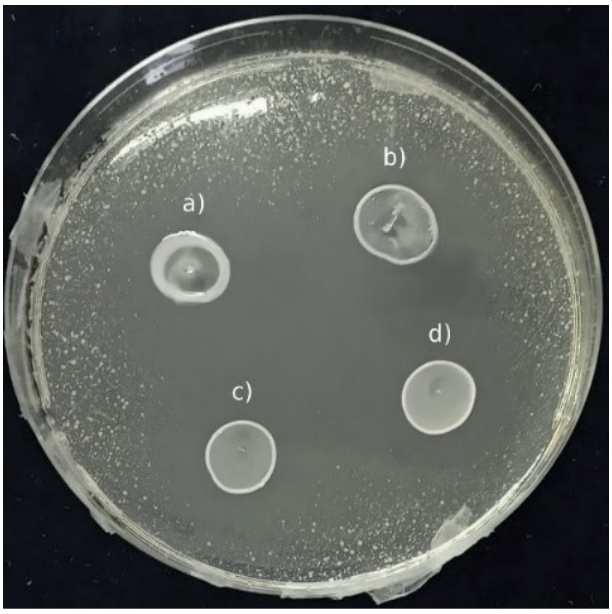

Figure 1. Inhibition activity of (a, b) *Lactiplantibacillus plantarum* 299v and (c, d) *Lactiplantibacillus plantarum* CCDM 181 against the yeast *Kluyveromyces lactis* var. *lactis* CCDM 271

inhibitory activity of lactic acid depending on the pH of the medium. A lactic acid concentration of 60 g L^{-1} was necessary to suppress it, while only 30 g L^{-1} was needed for *K. lactis*. The tested strains did not produce sufficient amounts of acids. A combination of the tested *L. plantarum* strains with yoghurt microorganisms was more effective in suppressing the yeast contamination caused by *K. marxianus* and *K. lactis*. It can be assumed that in combination with other LAB, sufficient suppression of yeast growth can occur.

When using *L. plantarum* in fermented products, their stability at low pH, compatibility with yoghurt culture strains and growth in selected media, i.e. milk and soy drink, are important. To select a suitable culture temperature, the growth ability of both strains at 30, 37, and 42 °C was measured as optical density (OD) (A_{600}) in MRS broth, pH 5.6. From the results summarised in Table 1, it is evident that the appropriate cultivation temperature is 30 °C or 37 °C; therefore, the industrially used overnight cultivation at 30 °C was chosen for the subsequent co-culturing with the yoghurt culture. Published studies also indicate suitable cultivation temperatures of 30 °C or 37 °C, with the fact that higher acidification activity may occur at higher temperatures (Wardani et al. 2017). In the study of Moreno-Montoro et al. (2018), they applied *L. plantarum* strains along with yoghurt culture to milk or plant beverage. Fermentation took place

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Table 1. Growth [as optical density $(OD)_{A600}$] of tested strains of *Lactiplantibacillus plantarum* at different temperatures in De Man, Rogosa and Sharpe (MRS) broth, pH 5.6 (mean ± SD; n = 3)

Strain	0 h	6 h	24 h
30 °C			
299v	0.007 ± 0.004	0.020 ± 0.003	2.217 ± 0.023
CCDM 181	0.001 ± 0.000	0.037 ± 0.004	2.012 ± 0.022
37 °C			
299v	0.007 ± 0.004	0.048 ± 0.005	2.038 ± 0.025
CCDM 181	0.001 ± 0.000	0.036 ± 0.007	2.024 ± 0.027
42 °C			
299v	0.007 ± 0.004	0.019 ± 0.003	1.436 ± 0.032
CCDM 181	0.001 ± 0.000	0.040 ± 0.001	0.484 ± 0.084

at 37 °C or 42 °C, but at the higher temperature, *L. plantarum* showed lower growth.

To study the effect of pH on lactobacilli, the measurement of growth curves in MRS broth at pH adjusted to 4, 5, or 6 was used. Both strains were able to grow well at all pH tested, although a lower growth rate was noted at pH 4 (data not shown).

Growth of *L. plantarum* strains and yoghurt culture in milk and soy beverage. For the preparation of the fermented product with yoghurt culture in combination with *L. plantarum*, skimmed UHT milk and soy beverage were selected as a medium. First, the growth of *L. plantarum* strains and yoghurt culture separately in these media during aerobic cultivation at 30 °C was verified. The results are shown in Table 2. The increase of the cell number of *L. plantarum* strains in milk was approximately 2 orders of magnitude. Authors testing different strains of *L. plantarum* concluded that milk was not a sufficiently rich medium for their growth (Wegkamp et al. 2010). The strains showed low acidifying activity in milk; a decrease from the initial value of pH 6.5 ± 0 to 6.3 ± 0.1 after 18 h of cultivation; this value did not change even after prolonged cultivation for 48 h. Milk has a strong buffering capacity; also other authors described only small changes in pH during the fermentation of milk by this species, e.g. Chengjie et al. (2016) reported a pH of 5.8 ± 0.1 after 72 h, but in media fortified with vitamins a pH of 4.0 ± 0.2 after 48 h cultivation. Due to the low acidifying activity of the tested strains in milk, the production of a fermented milk product without the use of another starter culture cannot be recommended. On the contrary, the unsweetened soy beverage provided better conditions for the growth of *L. plantarum* strains; there was an increase in the number of cells by about 3.5 orders of magnitude and a change in pH from the initial value of 7.3 ± 0.1 to the final pH 5.3 after 18 h cultivation. The species *L. plantarum* is more often found on plant substrates, which may also be the reason for its good adaptation to plant beverages (Chengcheng et al. 2014).

The growth of the yoghurt culture YC-381 in milk was standard, and in turn, it provided sufficient acid production to lower the pH below the isoelectric point of casein. Different growth of yoghurt culture microorganisms in the soy drink was interesting; with *S. thermophilus* there was an increase by 2 orders of magnitude, while with *L. delbrueckii* subsp. *bulgaricus* by only 1 order. This fact may be related to the different activity of α-galactosidase, which was not, however, determined in this study for the yoghurt culture. Nevertheless, Donkor et al. (2007) pointed to different utilisation of raffinose contained in soy drink by yoghurt microorganisms.

Co-culturing *L. plantarum* and yoghurt culture and compatibility of these microorganisms. The re-

Table 2. The number of cells and pH after cultivation of *Lactiplantibacillus plantarum* strains and yoghurt culture in milk and soy beverage at 30 °C (mean ± SD; n = 3)

Strain	Milk			Soy beverage		
	number of cells (log CFU mL^{-1})		pH	number of cells (log CFU mL^{-1})		pH
	0 h	18 h	18 h	0 h	18 h	18 h
Lactiplantibacillus plantarum 299v	6.4 ± 0.1	8.2 ± 0.4	6.3 ± 0.1	6.5 ± 0.4	10.0 ± 0.2	5.3 ± 0.4
Lactiplantibacillus plantarum CCDM 181	6.1 ± 0.0	8.1 ± 0.5	6.3 ± 0.1	6.4 ± 0.2	10.0 ± 0.2	5.2 ± 0.3
Yoghurt culture						
Streptococcus thermophilus	7.7 ± 0.1	9.9 ± 0.1	4.1 ± 0.1	7.7 ± 0.2	9.5 ± 0.1	4.7 ± 0.1
Lactobacillus delbrueckii	6.9 ± 0.0	9.5 ± 0.1	4.1 ± 0.1	6.8 ± 0.1	7.9 ± 0.1	4.7 ± 0.1

CFU – colony forming unit

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Table. 3. The number of cells of *L. plantarum* strains and yoghurt culture at the beginning, after co-culturing (30 °C, 18 h, aerobically) and after storage at 5 ± 1 °C in yoghurt and soy fermented product (CFU mL^{-1}) (mean ± SD; n = 2)

Strain	Yoghurt			Soy fermented product		
	inoculation (0 h)	after 18 h	28 days storage	inoculation (0 h)	after 18 h	28 days storage
L. plantarum 299v	7.8 ± 0.1	8.9 ± 0.0	7.3 ± 0.0	7.9 ± 0.1	10.1 ± 0.1	8.5 ± 0.0
S. thermophilus YC-381	7.2 ± 0.1	9.4 ± 0.1	7.6 ± 0.0	7.2 ± 0.1	8.5 ± 0.1	7.0 ± 0.0
L. delbrueckii subsp. *bulgaricus* YC-381	6.9 ± 0.1	9.4 ± 0.0	8.2 ± 0.0	6.9 ± 0.1	6.8 ± 0.1	4.9 ± 0.0
L. plantarum 181	7.8 ± 0.1	8.2 ± 0.1	7.6 ± 0.2	7.9 ± 0.1	10.0 ± 0.1	8.2 ± 0.2
S. thermophilus YC-381	7.1 ± 0.1	9.7 ± 0.1	8.0 ± 0.1	7.1 ± 0.1	8.7 ± 0.1	7.6 ± 0.0
L. delbrueckii subsp. *bulgaricus* YC-381	7.2 ± 0.0	9.6 ± 0.1	7.9 ± 0.0	7.2 ± 0.2	7.6 ± 0.1	5.3 ± 0.1

L. plantarum – Lactiplantibacillus plantarum; *S. thermophilus – Streptococcus thermophilus*; *L. delbrueckii – Lactobacillus delbrueckii*; CFU – colony forming unit

sults of the cell number of individual species during co-culturing in milk and soy beverage at 30 °C for 18 h and subsequent storage at 5 ± 1 °C are shown in Table 3. It is evident that different results were obtained from those of the separate cultivation of the strains, namely in the yoghurt culture and especially in the case of *L. delbrueckii* subsp. *bulgaricus*. During co-culturing in the soy drink, there was no increase of this microorganism (almost unchanged values comparing time 0 h and 18 h), and during storage, there was a decrease of cells by approximately 2 orders of magnitude. The increase was also lower for *S. thermophilus*. In the case of co-culturing with *L. plantarum,* the cell concentration increased by only 1 order of magnitude. These results are not consistent with some studies that reported that *L. plantarum* promotes the growth of *S. thermophilus* (Turchi et al. 2017). Moreno-Montoro et al. (2018) found no negative interactions between *L. plantarum* C4 and *S. thermophilus* YO-350, but they confirmed the growth suppression of *L. delbrueckii* subsp. *bulgaricus* YO-350. For yoghurts, similar cell count results were obtained after co-culturing, like with the cultivation of individual strains. After storage, there was a loss of cells, but they reached sufficient concentrations required for yoghurt culture, which is 10^7 CFU g^{-1}. For *L. plantarum*, the yoghurt and soy fermented product had a cell content of more than 10^7 CFU mL^{-1} and more than 10^8 CFU mL^{-1}, respectively. However, a reduction in the cell number of *L. plantarum* was significant (by 2 orders) during storage and was confirmed in other studies (Chengcheng et al. 2014). These results all together showed that *L. plantarum* strains proliferated successfully at low pH. This resistance may, of course, be a variable feature within the species.

Based on the results shown in Table 3, the compatibility of *L. plantarum* strains and yoghurt culture microorganisms was verified by the agar diffusion method. In addition to the strains used in the previous part of the study, other strains were also tested in order to demonstrate the strain specificity of this phenomenon. The inhibitory activity against *L. delbrueckii* was monitored on MRS soft agar and against *S. thermophilus* both on MRS soft agar and on M17 agar. The results are presented in Table 4 for *L. delbrueckii* subsp. *bulgaricus* and in Table 5 for *S. thermophilus*. An example of the agar

Table 4. Inhibition zone around colonies of *Lactiplantibacillus plantarum* in the agar diffusion method with *Lactobacillus delbrueckii* subsp. *bulgaricus* strain in De Man, Rogosa and Sharpe (MRS) agar (mm) (mean ± SD; n = 3)

L. plantarum strain	*L. delbrueckii* subsp. *bulgaricus*			
	YC 381	CCDM 31	CCDM 171	CCDM 25
299v	11.1 ± 0.4	12.6 ± 0.5	24.5 ± 0.8	19.6 ± 0.5
CCDM 181	10.3 ± 0.4	12.8 ± 0.4	24.4 ± 0.8	18.9 ± 0.7
ATCC 14917	11.9 ± 0.3	13.9 ± 0.3	24.3 ± 0.8	18.3 ± 1.1
CCDM 375	11.7 ± 0.4	12.8 ± 0.2	25.1 ± 0.2	19.1 ± 0.5

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Table 5. Inhibition zone around colonies of *Lactiplantibacillus plantarum* in the agar diffusion method with *Streptococcus thermophilus* strains in De Man, Rogosa and Sharpe (MRS) or M17 agar (mm) (mean ± SD; n = 3)

L. plantarum strain	*S. thermophilus*					
	YC 381		CCDM 31		CCDM 148	
	MRS	M17	MRS	M17	MRS	M17
299v	11.2 ± 1.1	7.8 ± 0.6	N	20.9 ± 2.8	20.9 ± 1.1	5.6 ± 0.5
CCDM 181	10.8 ± 1.2	7.2 ± 0.6	N	13.8 ± 0.6	23.3 ± 0.5	9.9 ± 0.5
ATCC 14917	13.0 ± 0.7	9.3 ± 0.5	N	6.6 ± 1.4	20.7 ± 0.8	0.0
CCDM 375	13.4 ± 0.3	8.2 ± 0.4	N	15.5 ± 2.1	22.4 ± 1.1	11.9 ± 1.1

N – no growth of *S. thermophilus* strain on MRS agar

diffusion method for *S. thermophilus* CCDM 31 is shown in Figure 2. The results proved the inhibitory activity of all *L. plantarum* strains on both microorganisms, the activity was strain-specific. In the case of *S. thermophilus*, the size of the inhibition zones was influenced by the selected cultivation medium. On M17 agar, which is intended and is more suitable for the cultivation of *S. thermophilus*, the zones were significantly smaller than when tested on MRS agar. The growth medium used can affect the production of organic acids or bacteriocins, which are the main antimicrobial substances produced by LAB (Zalán et al. 2010). A higher inhibitory activity against *L. delbrueckii* was also confirmed, which was also recorded during co-culturing in milk and soy beverage. The better growth ability of *L. plantarum* and the worse one of *L. delbrueckii* in soy medium, together with inhibitory activity, therefore, led to the suppression of its growth compared to growth in milk. Milk is more suitable for the growth of yoghurt culture and, conversely, less suitable for *L. plantarum*.

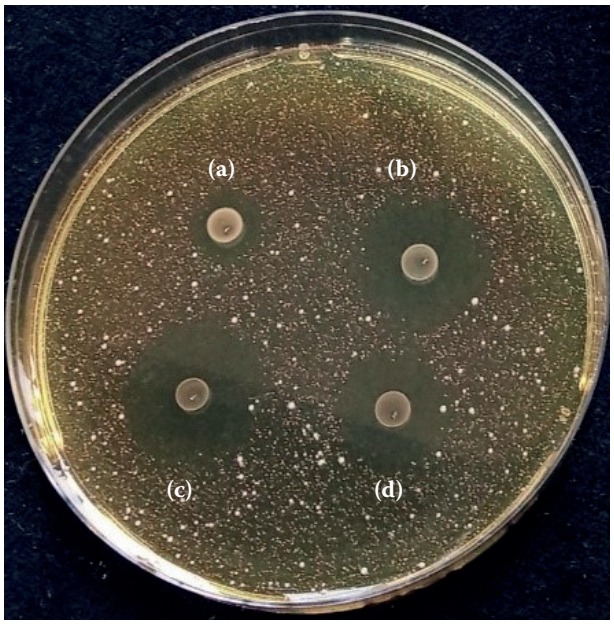

Figure 2. Inhibition zone detected by agar diffusion method around the spot of *Lactiplantibacillus plantarum* strain during cultivation with *Streptococcus thermophilus* CCDM 31 on M17 agar at 37 °C, 48 h cultivation: (a) *L. plantarum* ATCC 14971, (b) *L. plantarum* CCDM 375, (c) *L. plantarum* 299v, and (d) *L. plantarum* CCDM 181

CONCLUSION

The tested strains of *L. plantarum* 299v and CCDM 181 showed sufficient growth in both milk and soy beverages and were stable at low pH of 4.5 for 28 days at 5 °C typical regime for fermented product storage. When combined with a yoghurt culture, sufficient acidification of these media can be achieved for the preparation of a fermented product. *L. plantarum* strains showed better growth in soy beverage compared to milk, but both fermented media can be good vehicles for beneficial strains of *L. plantarum* with probiotic or protective activity into our diet. The growth of yoghurt culture was influenced by *L. plantarum* depending on the medium used. Due to the possible inhibitory activity against the microorganisms of the yoghurt culture, which is strain-specific, prior verification of the mutual compatibility of the strains for co-culture is necessary. *L. plantarum* strains inhibited more the growth of *L. delbrueckii* in the agar diffusion method, which, combined with its insufficient growth in the soy beverage, resulted in lower concentrations of this microorganism in the soy product compared to yoghurt.

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